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<p>(21) International Application Number: PCT/US97/00758 (22) International Filing Date: 17 January 1997 (17.01.97) (30) Priority Data: 08/587,458 17 January 1996 (17.01.96) US (60) Parent Application or Grant (63) Related by Continuation US 08/587,458 (CIP) Filed on 17 January 1996 (17.01.96) (71) Applicant (for all designated States except US): PROGENICS PHARMACEUTICALS, INC. [US/US]; 777 Old Saw Mill River Road, Tarrytown, NY 10591 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): LITWIN, Virginia, M. [US/US]; 205 Canopus Hollow Road, Putnam Valley, NY 10579 (US). ALLAWAY, Graham, P. [GB/US]; 1778 Hor- ton Avenue, Mohegan Lake, NY 10547 (US). MADDON, Paul, J. [US/US]; Apartment 25C, 60 Haven Avenue, New York, NY 10032 (US).</p>	<p>(74) Agent: WHITE, John, P.; Cooper &amp; Dunham L.L.P., 1185 Avenue of the Americas, New York, NY 10036 (US). (81) Designated States: AU, CA, JP, US, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the</i> <i>claims and to be republished in the event of the receipt of</i> <i>amendments.</i></p>	
<p>(54) Title: COMPOUNDS CAPABLE OF INHIBITING HIV-1 INFECTION (57) Abstract  This invention provides an antibody capable of specifically inhibiting the fusion of an HIV-1 envelope glycoprotein+ cell with an appropriate CD4+ cell without cross reacting with the HIV-1 envelope glycoprotein or CD4 and capable of inhibiting infection by one or more strains of HIV-1. This antibody is then used to identify a molecule which is important for HIV infection. Different uses of the antibody and the molecule are described.</p>		

Applicants: Olson and Maddon  
Serial No. : 09/828/615  
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**COMPOUNDS CAPABLE OF INHIBITING HIV-1 INFECTION**

5 This application is a continuation-in-part of U.S. Serial No. 08/587,458, filed January 17, 1996, the contents of which are incorporated herein by reference.

**Background of the Invention**

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Throughout this application, various references are referred to within parentheses. Disclosures of these publications in their entirety are hereby incorporated by reference into this application to more fully describe the state of the art to which this invention pertains. Full bibliographic citation for these references may be found at the end of this application, preceding the claims.

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This invention comprises a series of new therapeutics and targets for therapeutic intervention in HIV-1 infection. Monoclonal antibodies have been identified that inhibit HIV-1 envelope-mediated membrane fusion and thereby inhibit virus infection. The antibodies were discovered by screening panels of monoclonal antibodies generated by immunizing mice with human cells. The screening was initially performed using a resonance energy transfer (RET) assay of HIV-1 envelope-mediated membrane fusion. Antibodies which inhibited in this assay were further screened for inhibitory activity in a HIV-1 infection assay.

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These inhibitory antibodies act by binding to molecules on the surface of cells, which are required for HIV-1 to fuse with and infect target cells. The molecules are either previously unidentified or their role in HIV-1 entry was previously unrecognized. The cell surface

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molecules are known as accessory molecules, since they are required for virus entry in addition to the HIV-1 receptor, CD4. While CD4 is required for HIV-1 attachment, the accessory molecules are required for the membrane fusion step of entry. These accessory molecules are generally expressed only on human cells, so HIV-1 does not infect non-human cells that have been engineered to express human CD4 (1,3,8,9). Moreover, several groups have shown that it is possible to complement these non-human CD4<sup>+</sup> cells by fusing them (using polyethylene glycol) with CD4<sup>+</sup> human cells, resulting in a heterokaryon which is a competent target for HIV-1 envelope-mediated membrane fusion (2,5).

As discussed above, it is generally accepted that accessory molecules are required for HIV-1 fusion. However, the precise nature of these co-receptors or accessory molecules has not yet been discerned. While some cell surface molecules have previously been implicated as fusion accessory molecules (7,10,11), their role has not been confirmed (4).

In some cases, the fusion accessory molecules are found on a subset of human CD4<sup>+</sup> cells and are required for infection by HIV-1 isolates with particular tropisms. For example, macrophage-tropic strains of HIV-1 such as HIV-1<sub>JR-FL</sub> may have different requirements for accessory molecules compared with T lymphotropic strains such as HIV-1<sub>LAI</sub>. This phenomenon is, in part, responsible for differences in tropism between HIV-1 strains.

The current invention includes the monoclonal antibodies and the hybridomas which secrete them; also their humanized equivalents, single chain antibodies or antigen binding fragments of the antibodies. These antibodies,

single chain antibodies or antibody fragments have value as immunotherapeutics or immunoprophylactics for HIV-1 infection. The invention also includes the genes encoding these antibodies, single chain antibodies and antibody fragments. Moreover, the invention includes the accessory molecules recognized by these monoclonal antibodies, or components of these accessory molecules and region(s) of HIV-1 gp120/gp41 that interact with these accessory molecule(s). In addition the invention includes the genes encoding the accessory molecules. The accessory molecules or their fragments have value as therapeutic or prophylactic agents to inhibit HIV-1 infection. They are also valuable as a basis for rationale drug design to identify inhibitors of HIV-1 infection. This invention provides transgenic animals comprising DNA encoding these accessory molecules or fragments thereof. These transgenic animals are useful as animal models of HIV-1 infection. The invention also includes the use of the antibodies and/or accessory molecules in drug screening assays to identify inhibitors of HIV-1 fusion. Finally, the invention includes the inhibitors identified using these drug screens.

### Summary of the Invention

This invention provides an antibody capable of specifically inhibiting the fusion of an HIV-1 envelope glycoprotein' cell with an appropriate CD4' cell without cross reacting with the HIV-1 envelope glycoprotein or CD4 and capable of inhibiting infection by one or more strains of HIV-1.

This invention also provides a pharmaceutical composition comprising the complete or a portion of the above-described antibody and a pharmaceutically acceptable carrier. This invention provides a method of inhibiting HIV-1 infection in a subject comprising administering an effective amount of the above pharmaceutical composition to the subject.

This invention also provides nucleic acid molecules encoding the complete or a portion of the light chain and the heavy chain protein of the above antibody. This invention also provides vectors comprising these nucleic acid molecules operably linked to a promoter of RNA transcription. This invention also provides host vector systems comprising one or more of these vectors in a suitable host cell.

This invention provides the molecule specifically recognized by the monoclonal antibody capable of specifically inhibiting the fusion of an HIV-1 envelope glycoprotein' cell with an appropriate CD4' cell without cross reacting with the HIV-1 envelope glycoprotein or CD4 and which capable of inhibiting infection by one or more strains of HIV-1. In an embodiment, this is a glycolipid molecule. In another embodiment, this is a polypeptide. This invention also provides an isolated

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nucleic acid molecule encoding the complete or a portion of this polypeptide. In a still further embodiment, the molecule is a multichain polypeptide molecule.

5 This invention also provides a soluble protein which comprises a portion of the polypeptide or the multichain polypeptide molecule. This invention also provides a pharmaceutical composition comprising an effective amount of the soluble protein to inhibit HIV-1 infection and a  
10 pharmaceutically acceptable carrier. This invention also provides a method of inhibiting HIV-1 infection in a subject comprising administering an effective amount of the above pharmaceutical composition to the subject.

15 This invention provides an isolated nucleic acid molecule encoding the complete or a portion of a polypeptide of the above multichain polypeptide molecule. This invention also provides a vector comprising the nucleic acid molecule encoding the complete or a portion of a  
20 polypeptide of the above multichain polypeptide molecule operably linked to a promoter of RNA transcription, and a host vector system comprising this vector in a suitable host cell.

25 This invention also provides a method for identifying inhibitors of HIV-1 infection comprising steps of: (a) contacting an effective amount of a compound with a system which contains HIV-1 gp120, HIV-1 gp41 or a fragment thereof with the molecule specifically  
30 recognized by the monoclonal antibody capable of specifically inhibiting the fusion of an HIV-1 envelope glycoprotein' cell with an appropriate CD4' cell without cross reacting with the HIV-1 envelope glycoprotein or CD4 and capable of inhibiting infection by one or more  
35 strains of HIV-1 under conditions permitting formation of



a complex between HIV-1 gp120, HIV-1 gp41 or a fragment thereof and the molecule, so as to inhibit such formation; and (b) determining the amount of complex formed; and (c) comparing the amount determined in step  
5 (b) with the control which is without the addition of the compound, a decrease in the complex formation indicating that the compound is capable of inhibiting HIV-1 infection.

10 This invention also provides the identified compound and a pharmaceutical composition comprising the identified compound and a pharmaceutically acceptable carrier. This invention provides a method of inhibiting HIV-1 infection in a subject comprising administering an effective amount  
15 of this pharmaceutical composition to the subject.

This invention provides a kit for identifying inhibitors of HIV-1 infection which comprises, in separate compartments: (a) purified HIV-1 gp120, HIV-1 gp41 or a  
20 fragment thereof; and (b) the molecule specifically recognized by the monoclonal antibody capable of specifically inhibiting the fusion of an HIV-1 envelope glycoprotein\* cell with an appropriate CD4\* cell without cross reacting with the HIV-1 envelope glycoprotein or  
25 CD4 and capable of inhibiting infection by one or more strains of HIV-1.

Finally, this invention provides a transgenic nonhuman animal which comprises an isolated DNA molecule encoding  
30 the molecule specifically recognized by the monoclonal antibody capable of specifically inhibiting the fusion of an HIV-1 envelope glycoprotein\* cell with an appropriate CD4\* cell without cross reacting with the HIV-1 envelope glycoprotein or CD4 and which inhibits infection by one  
35 or more strains of HIV-1. This invention also provides

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the above-described transgenic nonhuman animal which further comprises a DNA molecule encoding human CD4. This invention further provides different uses of the transgenic animals for screening and development of HIV-1 drugs.

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### Brief Description of the Figures

Figure 1. Flow cytometric analysis of human T lymphoblastoid cell lines with mAb PA-3, PA-5, PA-6 and PA-7. Cells were incubated for 15 min at 4°C with 100 $\mu$ l of the indicated hybridoma supernatant (open histograms), then washed 3 times in PBS containing 0.05% NaN<sub>3</sub>. Following a second incubation with FITC-conjugated goat anti-mouse antibody and additional washes the cells were analyzed by flow cytometry. Controls (shaded histograms) were stained with FITC-conjugated goat anti-mouse antibody only. Fluorescence intensity is shown on the X-axis (four decade log scale) and the relative number of cells on the Y-axis. Columns 1-4 are stained with mAb PA-3, PA-5, PA-6 and PA-7, respectively. Rows 1-4 represent the cell lines PM-1, HUT 78, CEM and SUP-T1, respectively.

Figure 2. Cell surface expression of antigens recognized by the mAb PA-3, PA-5, PA-6 and PA-7 on CD4-transfected cell lines and non-transfected counterparts. A comparison of cell surface staining with PA-3, PA-5 and PA-6 and PA-7 was conducted on HeLa or HeLa-CD4 cells (Fig.2A-2E) and the Chinese hamster ovary cell line, DG44 or DG44 transfected with human CD4, DG44#3 (Fig.2F-2J). The CD4 expression on the transfectants and parental cells was demonstrated by staining with the CD4 specific mAb OKT4A. The histograms showing cell surface staining with monoclonal antibodies PA-3, PA-5, PA-6 and PA-7 on HeLa and HeLa-CD4 overlap,

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indicating that these antibodies do not recognize human CD4 nor do they recognize any other antigens expressed on the surface of HeLa Cells. Similarly when DG44 and DG44#3 are stained with monoclonal antibodies PA-3, PA-5, PA-6 and PA-7, the histograms overlap, indicating that these monoclonal antibodies do not recognize human CD4 or other surface antigens expressed on the DG44 cells. Although there is a slight increase in binding of PA-5 on the DG44#3 cells compared to the DG44 cells, this is not due to CD4 recognition but rather to non-specific binding. That PA-5 does not recognize human CD4 is confirmed by ELISA assay, staining with HeLa-CD4 cells and immunoprecipitation analysis (see figure 3). Staining and Axes are as described in Figure 1.

**Figure 3. Monoclonal antibodies PA-3, PA-5, VL125-6D1 recognize the same antigen.** PM-1 cells were surface labeled with biotin and immunoprecipitated with hybridoma supernatants PA-3, PA-5, VL125-6D1 and the CD4 specific antibody OKT4A (Ortho Diagnostics, Raritan, New Jersey) as described in the experimental methods. Precipitated antigens were resolved on a 4-15% gradient polyacrylamide gel under reducing conditions. Gels were scanned using a Molecular Dynamics (Sunnyvale, CA) densitometer. Molecular weight markers are as indicated in the far left lane. Monoclonal antibodies PA-3, PA-5 and VL125-6D1 precipitate two proteins of molecular weights approximately 158Kd and 87Kd.

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Figure 4. Antigens recognized by PA-3 but not PA-7 co-migrate with LFA-1 (CD11a/CD18). PM-1 cells were surface labeled with biotin and immunoprecipitated with hybridoma supernatants PA-3 and PA-7 and the CD3 specific UCHT1 (Pharmingen, San Diego, CA), the CD11a specific 25.3.1 (Immunotech, Westbrook, ME) and the CD18 specific 7E4 (Immunotech) as described in the experimental methods. Precipitated antigens were resolved on a 4-15% gradient polyacrylamide gel under reducing conditions. Gels were scanned using a Molecular Dynamics (Sunnyvale, CA) densitometer. Antigens precipitated by PA-3 but not PA-7 co-migrate with those precipitated by 25.3.1 and 7E4. Antigens recognized by UCHT1 did not resolve on this gel. Molecular weight markers are as indicated in the far left lane.

Figure 5. Monoclonal antibodies PA-6 and PA-7 recognize the same antigen. PM-1 cells were surface labeled with biotin and immunoprecipitated with hybridoma supernatants PA-3, PA-6, PA-7 and the CD4 specific antibody OKT4A (Ortho Diagnostics) as described in the experimental methods. Precipitated antigens were resolved on a 4-15% gradient polyacrylamide gel under reducing conditions. Gels were scanned using a Molecular Dynamics (Sunnyvale, CA) densitometer. Molecular weight marker are as indicated in the far left lane. Monoclonal antibodies PA-6 and PA-7, precipitate two proteins of molecular weights approximately 24.8Kd and 19.1Kd.

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Figure 6. Antigens recognized by PA-7 co-migrate with HLA class II. PM-1 cells were surface labeled with biotin and immunoprecipitated with hybridoma supernatants PA-7 and the CD4 specific OKT4A (Ortho) and the HLA class II specific TU39 (Pharmingen) as described in the methods section. Precipitated antigens were resolved on a 4-15% gradient polyacrylamide gel under reducing conditions. Antigens precipitated by PA-7 appear to co-migrate with those precipitated by TU39. Molecular weight markers are as indicated in the far left lane.

## Detailed Description of the Invention

This invention provides an antibody capable of specifically inhibiting the fusion of an HIV-1 envelope glycoprotein' cell with an appropriate CD4' cell without cross reacting with the HIV-1 envelope glycoprotein or CD4 and capable of inhibiting infection by one or more strains of HIV-1. In an embodiment, the antibody is a monoclonal antibody. This invention also provides a hybridoma cell line producing the monoclonal antibody.

In another embodiment, the antibody is a chimeric monoclonal antibody. In a separate embodiment, the antibody is a humanized monoclonal antibody. In a still separate embodiment, the antibody is a human monoclonal antibody.

For the purposes of this invention, a "chimeric" monoclonal antibody is a murine monoclonal antibody comprising constant region fragments ( $F_c$ ) from a different species. In a preferred embodiment of this invention, the chimeric monoclonal antibody comprises human  $F_c$  and murine  $F_{ab}$ . For the purposes of this invention, a "humanized" monoclonal antibody is a murine monoclonal antibody in which human protein sequences have been substituted for all the murine protein sequences except for the murine complementarity-determining regions (CDR) of both the light and heavy chains.

This invention also provides single chain antibodies or an antigen binding antibody fragment capable of specifically inhibiting the fusion of an HIV-1 envelope glycoprotein' cell with an appropriate CD4' cell without cross reacting with the HIV-1 envelope glycoprotein or CD4 and capable of inhibiting infection by one or more

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strains of HIV-1. The fragments include but are not limited to Fab and Fab'. The methods to generate the single chain antibodies and antigen binding antibody fragments with particular binding activities are well-known in the art (See for example, Crawley, P. (1995) Antibody Patents, in Monoclonal Antibodies: Principles and Applications, Wiley-Liss, Inc. NY, pages 299-335).

This invention also provides a monoclonal antibody capable of competitively inhibiting the binding of the monoclonal antibody capable of specifically inhibiting the fusion of an HIV-1 envelope glycoprotein' cell with an appropriate CD4' cell without cross reacting with the HIV-1 envelope glycoprotein or CD4 and capable of inhibiting infection by one or more strains of HIV-1 to its target molecule.

In one embodiment of this invention, the monoclonal antibody is labelled with a detectable marker, for example, a radioactive isotope, enzyme, dye or biotin. This invention provides a pharmaceutical composition comprising the complete or a portion of the monoclonal antibody capable of specifically inhibiting the fusion of an HIV-1 envelope glycoprotein' cell with an appropriate CD4' cell without cross reacting with the HIV-1 envelope glycoprotein or CD4 and capable of inhibiting infection by one or more strains of HIV-1 and a pharmaceutically acceptable carrier.

For the purposes of this invention "pharmaceutically acceptable carriers" means any of the standard pharmaceutical carriers. Examples of suitable carriers are well known in the art and may include, but are not limited to, any of the standard pharmaceutical carriers such as phosphate buffered saline solutions, emulsions



such as oil/water emulsion, and various type of wetting agents. Other carriers may also include sterile solutions, tablets, coated tablets, and capsules.

5 Typically such carriers contain excipients such as starch, milk, sugar, certain types of clay, gelatin, stearic acid or salts thereof, magnesium or calcium stearate, talc, vegetable fats or oils, gums, glycols, or  
10 other known excipients. Such carriers may also include flavor and color additives or other ingredients. Compositions comprising such carriers are formulated by well known conventional methods.

15 The monoclonal antibodies described and claimed herein are useful for isolating the compound to which the monoclonal antibodies bind.

This invention provides a method of inhibiting HIV-1 infection in a subject comprising administering an  
20 effective amount of the above-described pharmaceutical composition to the subject.

This invention provides an isolated nucleic acid molecule encoding the complete or a portion of the light chain of  
25 the monoclonal antibody. In one embodiment of this invention, the nucleic acid molecule is a DNA molecule. In another embodiment, the DNA molecule is a cDNA molecule. In a separate embodiment, the DNA molecule is a genomic DNA molecule.

30 This invention provides an isolated nucleic acid molecule encoding the complete or a portion of the heavy chain of the monoclonal antibody. In one embodiment of this invention, the nucleic acid molecule is a DNA molecule.  
35 In another embodiment, the DNA molecule is a cDNA.

molecule. In a separate embodiment, the DNA molecule is a genomic DNA molecule.

5 This invention provides an isolated nucleic acid molecule encoding the above-described single chain antibody.

The DNA molecules of the subject invention also include DNA molecules coding for polypeptide analogs, fragments or derivatives of polypeptides which differ from  
10 naturally-occurring forms in terms of the identity or location of one or more amino acid residues (deletion analogs containing less than all of the residues specified for the protein, substitution analogs wherein one or more residues specified are replaced by other  
15 residues and addition analogs wherein one or more amino acid residues is added to a terminal or medial portion of the polypeptides) and which share some or all of the properties of the naturally-occurring forms. These include: the incorporation of codons "preferred" for  
20 expression by selected non-mammalian hosts; the provision of sites for cleavage by restriction endonuclease enzymes; and the provision of additional initial, terminal or intermediate DNA sequences that facilitate construction of readily expressed vectors.

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The nucleic acid sequences described and claimed herein are useful for generating new viral and circular plasmid vectors described below.

30 This invention provides the vector, for example a plasmid or a viral vector, comprising a nucleic acid molecule encoding the complete or a portion of light chain protein of the monoclonal antibody operably linked to a promoter of RNA transcription. This invention also provides a  
35 vector, for example a plasmid or a viral vector,

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comprising a nucleic acid molecule encoding the complete or a portion of heavy chain protein of the monoclonal antibody operably linked to a promoter of RNA transcription.

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This invention also provides a vector comprising a nucleic acid molecule encoding the above-described single chain antibody operably linked to a promoter of RNA transcription.

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This invention also provide a vector comprising the nucleic acid molecules encoding the complete or a portion of light chain protein and the complete or a portion of heavy chain protein of the monoclonal antibody each operably linked to a promoter of RNA transcription.

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This invention also provide a host vector system comprising one or more vectors which comprise either the complete or portion of the light chain or a complete or portion of the heavy chain or a combination thereof in a suitable host cell.

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This invention further provides the above host vector system, wherein the suitable host cell is selected from a group consisting of a bacterial cell, an insect cell, a yeast cell or a mammalian cell.

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This invention provides the molecule specifically recognized by the monoclonal antibody capable of specifically inhibiting the fusion of an HIV-1 envelope glycoprotein\* cell with an appropriate CD4\* cell without cross reacting with the HIV-1 envelope glycoprotein or CD4 and capable of inhibiting infection by one or more strains of HIV-1. In an embodiment, the molecule is a glycolipid. In another embodiment, the molecule is a

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polypeptide.

5 This invention also provides an isolated nucleic acid molecule encoding the complete or a portion of the polypeptide which is specifically recognized by the monoclonal antibody capable of specifically inhibiting the fusion of an HIV-1 envelope glycoprotein\* cell with an appropriate CD4\* cell without cross reacting with the HIV-1 envelope glycoprotein or CD4 and capable of  
10 inhibiting infection by one or more strains of HIV-1.

15 This invention provides a multichain polypeptide molecule comprising the polypeptide which is specifically recognized by the monoclonal antibody capable of specifically inhibiting the fusion of an HIV-1 envelope glycoprotein\* cell with an appropriate CD4\* cell without cross reacting with the HIV-1 envelope glycoprotein or CD4 and capable of inhibiting infection by one or more strains of HIV-1.

20 This invention also provides an isolated nucleic acid molecule encoding the complete or a portion of a polypeptide of the above-described multichain polypeptide molecule. This invention also provides vectors  
25 comprising the nucleic acid molecule encoding the complete or a portion of a polypeptide of the above-described multichain polypeptide molecule operably linked to a promoter of RNA transcription. This invention also provides vectors comprising the nucleic acid molecule  
30 encoding the complete or a portion of the above polypeptide molecule operably linked to a promoter of RNA transcription.

35 This invention also provides a host vector system comprising the above vectors in a suitable host cell.

The suitable host cell includes but is not limited to a bacterial cell, an insect cell, a yeast cell, a plant cell or a mammalian cell.

5 The above-described molecules specifically recognized by the antibody may be used to vaccinate healthy subjects for prevention or therapy of HIV infection. These molecules are also useful in identifying specific sites to which these molecules bind on the HIV-1 envelope glycoproteins gp120 and gp41. The sites revealed may be used to generate more specific antibodies directed to these sites. Moreover, this information may be used to design drugs which inhibit the binding of the HIV-1 envelope glycoproteins gp120 or gp41 to the molecule.

15 This invention also provides a soluble protein which comprises a portion of the polypeptide or the multichain polypeptide molecule.

20 For the purposes of this invention, a "soluble protein" is a protein free of cell membranes and other cellular components. In one embodiment of this invention, the soluble protein is labelled with a detectable marker, for example, a radioactive isotope, enzyme, dye or biotin. The soluble protein is valuable as a product for making a new and useful pharmaceutical composition.

30 This invention also provides a pharmaceutical composition comprising an effective amount of the above soluble protein to inhibit HIV-1 infection and a pharmaceutically acceptable carrier.

35 Methods of determining an "effective amount" are well known to those skilled in the art. Simple titration experiments using different amounts of the soluble

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protein administered to different animal models of HIV-1 infection or to HIV-1 infected human subjects may be performed to determine such an effective amount. The amount administered to the animal model of HIV-1 infection or HIV-1 infected humans which results in a reduction in HIV-1 infection is an effective amount.

This invention also provides a method of inhibiting HIV-1 infection in a subject comprising administering an effective amount of the pharmaceutical composition of the above soluble protein to the subject.

For the purposes of this invention, "administration" means any of the standard methods of administering a pharmaceutical composition known to those skilled in the art. Examples include, but are not limited to, intravenous, intraperitoneal or intramuscular administration.

This invention provides an isolated nucleic acid molecule encoding the soluble protein. In one embodiment of this invention, the nucleic acid molecule is a DNA molecule. Preferably, the DNA molecule is a cDNA molecule.

The nucleic acid sequences which encode the soluble protein are useful for generating new viral and circular plasmid vectors described below. The nucleic acid molecules are valuable in a new and useful method of gene therapy, i.e., by stably transforming cells isolated from a patient with the nucleic acid molecules and then readministering the stably transformed cells to the patient. Methods of isolating cells include any of the standard methods of withdrawing cells from an animal. Suitable isolated cells include, but are not limited to, bone marrow cells. Methods of readministering cells

include any of the standard methods of readministering cells to a patient. United States Patent 5,366,346, entitled, "Gene Therapy" describes different gene therapy procedures, the contents of which are incorporated herein by reference.

This invention also provides a vector, for example, a plasmid vector or a viral vector, comprising the isolated nucleic acid molecule operably linked to a promoter of RNA transcription.

The vectors described and claimed herein are valuable as products useful for generating stably transformed eukaryotic host cells, and thereby in new and useful methods of growing such host cells under conditions suitable for the production of the soluble protein.

This invention further provides a host vector system comprising the vector having the sequence which encodes the soluble protein in a suitable host cell. In one embodiment of this invention, the suitable host cell is a stably transformed eukaryotic cell, for example, a stably transformed eukaryotic yeast or mammalian cell. Preferably, the stably transformed cell is a mammalian cell.

The host vector system is valuable as a product useful for the large scale synthesis of the soluble protein by growing the host vector system under conditions suitable for the production of protein and recovering the protein so produced. Thus, a method of producing the soluble protein is also provided. This invention further provides the soluble protein produced by this method.

This invention provides a method for identifying

inhibitors of HIV-1 infection comprising steps of: (a) contacting an effective amount of a compound with a system which contains HIV-1 gp120, HIV-1 gp41 or a fragment thereof with the molecule specifically recognized by the monoclonal antibody capable of specifically inhibiting the fusion of an HIV-1 envelope glycoprotein' cell with an appropriate CD4' cell without cross reacting with the HIV-1 envelope glycoprotein or CD4 and capable of inhibiting infection by one or more strains of HIV-1 under conditions permitting formation of a complex between the HIV-1 gp120, HIV-1 gp41 or a fragment thereof with the molecule, so as to inhibit such formation; (b) determining the amount of complex formed; and (c) comparing the amount determined in step (b) with the control which is without the addition of the compound, a decrease in the complex formation indicating that the compound is capable of inhibiting HIV-1 infection. In an embodiment, the compound tested is not previously known. This invention also provide the compound identified by the above method.

This invention also provides a pharmaceutical composition comprising the compound identified by the above method and a pharmaceutically acceptable carrier.

This invention provides a method of inhibiting HIV-1 infection in a subject comprising administering an effective amount of the above pharmaceutical composition comprising the compound identified by the above method to the subject.

This invention provides a kit for identifying inhibitors of HIV-1 infection which comprises, in separate compartments: (a) purified HIV-1 gp120, HIV-1 gp41 or a fragment thereof; and (b) the molecule specifically



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recognized by the monoclonal antibody capable of specifically inhibiting the fusion of an HIV-1 envelope glycoprotein' cell with an appropriate CD4' cell without cross reacting with the HIV-1 envelope glycoprotein or CD4 and capable of inhibiting infection by one or more strains of HIV-1.

This invention provides a transgenic nonhuman mammal which comprises an isolated DNA molecule encoding a molecule specifically recognized by the monoclonal antibody capable of specifically inhibiting the fusion of an HIV-1 envelope glycoprotein' cell with an appropriate CD4' cell without cross reacting with the HIV-1 envelope glycoprotein or CD4 and capable of inhibiting infection by one or more strains of HIV-1.

One means available for producing a transgenic animal, with a mouse as an example, is as follows: Female mice are mated, and the resulting fertilized eggs are dissected out of their oviducts. The eggs are stored in an appropriate medium such as M2 medium (Hogan B. et al. Manipulating the Mouse Embryo, A Laboratory Manual, Cold Spring Harbor Laboratory (1986)). DNA or cDNA encoding the molecule specifically recognized by the monoclonal antibody capable of specifically inhibiting the fusion of an HIV-1 envelope glycoprotein' cell with an appropriate CD4' cell without cross reacting with the HIV-1 envelope glycoprotein or CD4 and capable of inhibiting infection by one or more strains of HIV-1 is purified from a vector by methods well known in the art. Inducible promoters may be fused with the coding region of the DNA to provide an experimental means to regulate expression of the transgene. Alternatively or in addition, tissue specific regulatory elements may be fused with the coding region to permit tissue-specific expression of the transgene.

The DNA, in an appropriately buffered solution, is put into a microinjection needle (which may be made from capillary tubing using a pipet puller) and the egg to be injected is put in a depression slide. The needle is inserted into the pronucleus of the egg, and the DNA solution is injected. The injected egg is then transferred into the oviduct of a pseudopregnant mouse (a mouse stimulated by the appropriate hormones to maintain pregnancy but which is not actually pregnant), where it proceeds to the uterus, implants, and develops to term. As noted above, microinjection is not the only method for inserting DNA into the egg cell, and is used here only for exemplary purposes.

CD4 may be expressed in the above transgenic animal system such that the animal will be susceptible to HIV-1 infection. Accordingly, this invention also provides the above transgenic nonhuman animals further comprising an isolated DNA molecule encoding the full-length or portion of the CD4 molecule sufficient for binding the HIV-1 envelope glycoprotein. These animal model systems are useful for screening compounds which are capable of inhibiting HIV-1 infection. Moreover, they are useful in predicting or evaluating possible therapeutic applications of HIV drugs. Therefore, this invention also provides a method for screening compounds using these transgenic animals.

This invention will be better understood from the Experimental Details which follow. However, one skilled in the art will readily appreciate that the specific methods and results discussed are merely illustrative of the invention as described more fully in the claims which follow thereafter.

## Experimental Details

This invention describes mAb which recognize accessory molecules required for HIV-1 membrane fusion and tropism. mAb to accessory molecules are first selected by the ability to inhibit HIV-1 envelope glycoprotein-mediated membrane fusion in the RET assay and then analyzed for the ability to inhibit HIV-1 infection in vitro.

## Methods

### Immunizations and hybridoma production.

The immunizing cell line is washed extensively in PBS. Five female six week old Balb/c mice (Charles River Laboratories) are immunized intraperitoneally (IP) with  $5 \times 10^6$  cells. Animals receive a minimum of two IP inoculations followed by a three week rest interval. Three days prior to splenectomy one animal receives an intravenous (IV) injection of  $0.5 \times 10^6$  cells. The additional animals continue to receive immunizations according to this schedule. Polyethylene glycol (Boehringer Mannheim, Indianapolis, IN) is used to fuse murine splenocytes with the murine myeloma cell line Sp2/0 to generate hybridoma cell lines. Hybridomas are plated in 96-well flat bottom tissue culture plates, selected in  $1 \mu\text{g/ml}$  azaserine (Sigma), 5 mM hypoxanthine (Boehringer Mannheim) and 0.8 mM thymidine (Boehringer Mannheim) and maintained in DMEM supplemented with 20% fetal calf serum (FCS), 2 mM L-glutamine, 0.1 mM non-essential amino acids, 1mM Na pyruvate, 100 U/ml penicillin, 100  $\mu\text{g/ml}$  streptomycin and 10 mM HEPES buffer. When a majority of the hybridomas reach confluency, generally in 10 to 14 days, 100  $\mu\text{l}$  of supernatant are removed and analyzed for the ability to inhibit HIV-1-mediated membrane fusion in the RET assay.

**RET Assay.**

The RET assay has been adapted to measure HIV-1 envelope glycoprotein-mediated membrane fusion mediated by the laboratory-adapted T cell-tropic strain HIV-1<sub>LAI</sub> and the macrophage-tropic clinical isolate HIV-1<sub>JR-FL</sub>. HeLa-env<sub>LAI</sub> cells are able to fuse with a variety of CD4-expressing cells; whereas, HeLa-env<sub>JR-FL</sub> cells are only able to fuse with PM1 cells, of the panel of cell lines tested.

Briefly, HeLa cells stably expressing HIV-1 gp120/gp41 are labeled with octadecyl fluorescein (F18) and mixed with octadecyl rhodamine (R18)-labeled CD4<sup>+</sup> target cells. Membrane fusion of the labeled cells is measured by exciting F18 and measuring emission from R18 (RET) which occurs only when the dyes are closely associated in the same membrane. At the initiation of the RET assay, 100  $\mu$ l of hybridoma cell supernatant is combined with  $2 \times 10^4$  F18-labeled HeLa-env cells and an equal number of R18-labeled CD4-expressing cells. Results from the RET assay are quantitatively determined using a fluorescence plate reader, data are transferred directly into a spreadsheet and the %RET and % inhibition of RET are automatically calculated. Hybridoma cell supernatants which achieve 50% inhibition or greater in the RET assay are selected for further analysis.

The percent inhibition of RET is defined as follows:

$$\% \text{ Inhibition} = (\text{Max RET} - \text{Exp RET}) / (\text{Max RET} - \text{Min RET}) \cdot 100$$

where Max RET is the %RET value obtained at four hours with HeLa-env cells and CD4-expressing cells in the absence of an inhibitory compound; Exp RET is the %RET value obtained for the same cell combination in the

presence of an inhibitory compound and Min RET is the background %RET value obtained using HeLa cells in place of HeLa envelope glycoprotein-expressing cells.

5 **Characterization and purification of the novel mAb.**

The novel mAb generated in this study are characterized. First mAb are tested for reactivity to human CD4. Any mAb found to be specific for human CD4 are not further analyzed and are not considered part of this invention.

10 The hybridoma cell lines which secrete mAb that do not react with CD4 are cloned and used for ascites production, and the mAb are isotyped and purified and tested for ability to inhibit HIV-1 infection.

15 **ELISA assay for the detection of CD4-specific mAb.**

Immulon 1 plates (Dynatech Laboratories, Chantilly, VA) are coated overnight with sCD4 (Progenics) at 120 ng/well in carbonate buffer at pH 9.4 at 4°C. Plates are then washed three times in PTB (PBS containing 0.5% Tween 20, 20 1% FCS and 0.1% BSA). Following blocking with 5% BSA and three additional washes, plates are incubated for one hour in the presence of 100 µl of hybridoma supernatant or a standard. The anti-CD4 mAb OKT4A is used as the standard and added at concentrations ranging from 60 25 ng/ml to 3.25 ng/ml. Plates are washed three times before and after the addition of 100 ng/well of horseradish peroxidase-conjugated goat anti-mouse (Kirkegaard and Perry Laboratories, Gaithersburg, MD).

30 O-phenylenediamine (OPD) at 1.2 mg/ml in solution (50 mM Na<sub>2</sub>HPO<sub>4</sub>, 25 mM citric acid and 1.2% H<sub>2</sub>O<sub>2</sub>) is then added and the plates developed in the dark for 10-20 min. The reaction is terminated by the addition of 100 µl of 1.3 M H<sub>2</sub>SO<sub>4</sub>. The A<sub>492</sub> is measured on a SLT 400 ATC plate 35 reader (Tecan US, Research Triangle Park, NC) and the

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data analyzed with the winSeLect software program (Tecan).

Cloning hybridoma cell lines by limiting dilution.

5 Hybridoma cell lines are cloned twice by the limiting dilution method. Cells are adjusted in hybridoma culture media to a concentration of five cells per ml by serial dilution. To a 96-well round bottom microliter plate, a volume of 200  $\mu$ l is pipetted, achieving a density of one  
10 cell per well. Supernatants are screened in the RET assay when the cells have reached confluency. Three clones from each of the original cell lines are selected based both on growth rate and the ability to inhibit HIV-1 envelope glycoprotein-mediated membrane fusion and  
15 are cryopreserved. The best of the three clones from each group is re-cloned by limiting dilution. Again, the three best clones are cryopreserved. One clone from each of the original hybridomas is selected for ascites production. Prior to ascites production, the isotype of  
20 the clones is determined.

Isotype determination.

The isotype of the monoclonal antibody secreted by each of the twice-cloned hybridoma cell lines is determined  
25 using the ImmunoType kit (Sigma). This is an ELISA-based method performed on nitrocellulose strips coated with anti-murine isotype specific antibody.

Ascites production

30 For each cloned hybridoma cell line, fifteen female six week old Balb/c mice (Charles River Laboratories) are primed with 0.5 ml of pristane (Sigma). Following a ten day rest period, mice are injected intraperitoneally with  $1 \times 10^6$  hybridoma cells. As ascites fluid accumulates the  
35 peritoneal cavity is drained and the fluid collected.

Antibody purification

Antibody is purified directly from ascites fluid using the protein A-based ImmunoPure Plus purification kit (Pierce, Rockford, IL). Ascites fluid is clarified by high speed centrifugation, is diluted and applied to a protein A agarose column. After the column has been extensively washed, antibody is eluted in a high pH buffer. Following neutralization and concentration, the  $A_{280}$  is measured and protein concentration determined.

Characterization of the molecules recognized by novel mAb.

The antigens recognized by the novel mAb are characterized. Flow cytometric analysis is conducted in order to evaluate the distribution of antigen expression in hematopoietic cell populations, in cell lines known to be HIV-1 membrane fusion competent or resistant, and in non-primate cell lines. A biochemical analysis of the antigens recognized by the novel mAb is performed. Candidate target epitopes include amino-acid epitopes on proteins, or carbohydrate epitopes on glycoproteins or glycolipids. These possibilities are distinguished by the following analyses. First, antigens are immunoprecipitated from cells in which the surface proteins have been labeled, followed by SDS-PAGE. To determine whether the mAb are recognizing carbohydrate epitopes, cells are treated with neuraminidase prior to FACS analysis and immunoprecipitation. In addition, inhibition of mAb binding to cells by lectins is examined. If these methods indicate the mAb do not recognize proteins but do recognize a carbohydrate epitope, thin layer chromatography is used to determine if the antigens are glycolipids.

Determination of cellular distribution of the molecules by flow cytometry.

The distribution of expression of the molecules in peripheral blood mononuclear cell (PBMC) populations is determined. The mononuclear cells are separated from peripheral blood using a Ficoll-Hypaque (Pharmacia) density gradient. PBMC are stained with the hybridoma cell supernatants and antigen expression in the lymphoid and myeloid populations determined by forward and side-scatter gating. The expression of the molecules on various cell lines is compared to the ability of these cell lines to fuse with HIV-1 envelope glycoprotein-expressing cells. The distribution of expression in non-primate cell lines is also evaluated.

Biochemical analyses

Immunoprecipitation. Immunoprecipitation analysis is used to determine whether proteins are recognized by the novel mAb. A non-radioactive immunodetection with ECL Western blotting (Amersham Life Science, Arlington Heights, IL) is used. In this method, cells are labeled with biotin NHS ester. The biotinylated cells are then subjected to lysis and immunoprecipitated with 100  $\mu$ l of hybridoma supernatant or control mAb. Following SDS-PAGE using 4-15% gradient gels and transfer to nitrocellulose, the immunoprecipitated antigens are visualized using streptavidin-HRP and enhanced chemiluminescence followed by exposure to X-ray film (Kodak). Biotin-labeled cells are lysed in 0.5% NP-40 Lysis Buffer (0.05 M Tris-HCl pH 8.0 containing 0.5% NP-40, 0.15 M NaCl, 0.02%  $\text{NaN}_3$ , 20 KIU/ml aprotinin, 1 mM PMSF). Immune complexes are precipitated with rabbit anti-mouse (Pel-Frez) coated pansorbin (Calbiochem, La Jolla, CA). Electrophoresis is performed under reducing and non-reducing conditions to determine if the molecules exist in a monomeric or



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complexed form. ECL protein molecular weight markers are used to estimate the molecular weights of the molecules.

If immunoprecipitation analysis indicates that the molecules recognized by the novel mAb are proteins, the carbohydrate modifications of these proteins are evaluated. To determine if the proteins contain sialic acid modifications, N-linked oligosaccharides or O-linked oligosaccharides, the immunoprecipitated-antigens are treated with neuraminidase (Sigma), N-glycanase (Boehringer Mannheim) and O-glycanase (Genzyme, Cambridge, MA), respectively. A comparison of the mobilities on SDS-PAGE of the enzyme-treated and untreated antigens is made.

#### Inhibition analysis of HIV-1 infection in vitro using novel mAb.

HIV-1 inhibition studies with the mAb are performed with a panel of diverse viruses, including both laboratory-adapted strains and primary isolates. Purified mAb are used in the infection experiments.

#### HIV-1 in vitro infection assay

Infection studies are performed with both laboratory-adapted strains and primary isolates of HIV-1. Initial experiments are performed with the laboratory adapted strain HIV-1<sub>NLA-3</sub> and primary isolate HIV-1<sub>JR-FL</sub>. First, the candidate mAb are pre-incubated for 30 min at 37°C with phytohemagglutinin (PHA)-activated human peripheral blood mononuclear cells (PBMC) prepared from HIV-1 seronegative donors. For each virus strain, an inoculum of approximately 50 TCID<sub>50</sub> is added to  $2 \times 10^6$  PBMC. The cultures are washed three times on day 1, the medium changed on day 4, and the cellular supernatants assayed for p24 core antigen expression using a

commercial kit (Abbott Laboratories) on day 7. For each viral strain, the p24 antigen concentrations in the mAb-treated cultures are compared with those of an untreated culture to determine the percentage inhibition of infection. CD4-IgG2, a fusion protein between CD4 and IgG2 which neutralizes all strains of HIV-1, is used as a positive control inhibitor. Initially, three concentrations (50, 25 and 12.5  $\mu\text{g/ml}$ ) of the candidate monoclonal antibodies are tested for the ability to inhibit infection by the primary isolate and laboratory-adapted strain of HIV-1. If inhibition is evident, a dose response relationship is determined with serial five-fold dilutions, starting at 50  $\mu\text{g/ml}$ , of the candidate mAb, CD4-IgG2 or other positive or negative control antibodies. A comparison of the p24 antigen concentration in the mAb-treated and untreated cultures is used to determine the percent neutralization,  $\text{IC}_{50}$  and  $\text{IC}_{90}$  values by linear regression analysis. The infection experiments are extended to include a larger panel of HIV-1 isolates if promising results are achieved in the preliminary experiments. Alternative target cell lines may be used in place of PBMCs, for example, PM1 cells may be used for HIV-1<sub>JR-FL</sub> infection experiments.

## Experimental Results

The following is a summary of the main experiments pertinent to this invention.

### Immunization with HeLa cells.

Applicants' initial strategy was to immunize with the human carcinoma cell line, HeLa, and screen for monoclonal antibodies (mAb) which inhibited HIV-1 envelope glycoprotein-mediated membrane fusion as detected in the RET assay using HeLa-env<sub>LAI</sub> and HeLa-CD4

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cells. Following the first fusion of splenocytes from the HeLa-immunized mice to the murine melanoma fusion partner, SP2/0, hybridoma supernatants were screened in the RET assay but no inhibitory mAb were identified.

5 This scheme was repeated twice more but no inhibitory mAb were identified.

#### **Immunization with C8166 cells.**

10 The next approach was to use the human CD4<sup>+</sup> T lymphoblastoid cell line C8166 for immunization, followed by screening with the RET assay using HeLa-env<sub>LAI</sub> and HeLa-CD4 cells. This strategy was repeated twice. Several hybridomas were identified which secreted mAb inhibiting HIV-1 envelope glycoprotein-mediated membrane fusion during the course of this analysis. All of these  
15 were later identified as CD4-specific mAb and thus are not included as part of this invention. However, this work did provide evidence that it is possible to produce mAb to cell surface molecules which inhibit fusion in the  
20 RET assay.

#### **Immunization with protease-digested human erythrocytes.**

25 The third strategy involved immunization with proteinase K digested human erythrocytes and screening with the RET assay using HeLa-env<sub>LAI</sub> and HeLa-CD4 cells. This approach was used following a published study which had found that when human erythrocytes were fused to mouse CD4<sup>+</sup> cells using polyethylene glycol, the heterokaryons became competent targets for HIV-1 envelope-mediated membrane  
30 fusion, suggesting that erythrocyte membranes contain fusion accessory molecules (6). Moreover, proteinase K treatment of the erythrocytes did not abrogate this complementation. Following immunization with proteinase K digested erythrocytes, applicants selected several  
35 hybridomas which inhibited in the RET assay using

HeLa-env<sub>LAI</sub> and HeLa-CD4 cells. Unfortunately, the hybridomas were unstable and thus failed to inhibit in subsequent RET assays.

5     **Immunization with PM1 cells.**

HeLa cells (HeLa-env<sub>JR-FL</sub>) which stably express the envelope glycoprotein from the JR-FL strain of HIV-1 were generated. The RET assay was then adapted to measure the fusion of HeLa-env<sub>JR-FL</sub> with PM1 cells. PM1 cells were  
10    then used for immunizations. The resulting hybridomas were screened in the RET assay using HeLa-env<sub>JR-FL</sub> and PM1 cells. Several hybridomas which inhibited fusion by 50% or greater were selected for further analysis. Hybridomas were analyzed by RET assay, flow cytometry and  
15    ELISA assay. All of the hybridomas selected for further analysis secreted mAb which continue to inhibit in the RET assay using HeLa-env<sub>JR-FL</sub> and PM1 cells. Four of these hybridomas designated PA-3, PA-5, PA-6 and PA-7, have been cloned twice by the limiting dilution method and  
20    ascites fluid has been produced. mAb are currently being purified from the ascites fluid.

25    **Inhibition of HIV-1 envelope glycoprotein-mediated membrane fusion in the RET assay by anti-PM1 hybridoma supernatants.**

In addition to inhibiting in the RET assay using HeLa-env<sub>JR-FL</sub> and PM1 cells, the culture supernatants from PA-3, PA-5, PA-6 and PA-7 also inhibit in the RET assay using HeLa-env<sub>LAI</sub> cells and certain CD4<sup>+</sup> target cells  
30    (Table 1). HIV-1<sub>LAI</sub> envelope glycoprotein-mediated membrane fusion with PM-1, and HUT-78 was inhibited by all of the mAb secreted from these hybridoma cell lines. Whereas, fusion between HeLa-env<sub>LAI</sub> and CEM was inhibited by PA-3 and PA-5 but less so by PA-6 or PA-7. The fusion  
35    between HeLa-env<sub>LAI</sub> and C8166 or Sup-T1 cells was

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inhibited minimally or not at all by these mAb.

Table 1. Inhibition of HIV-1 envelope glycoprotein mediated cell fusion by novel mAb.

5	Envelope expressing cells	CD4+ cells	% RET	% Inhibition of RET by novel mAb			
				PA-3	PA-5	PA-6	PA-7
10	HeLa-env <sub>JR-FL</sub>	PM-1	16.3	85.3	96.3	92	67
	HeLa-env <sub>LAI</sub>	PM-1	12.4	89.7	100	81	69
	HeLa-env <sub>LAI</sub>	HUT-78	10.9	51.3	60.3	55.7	52.7
	HeLa-env <sub>LAI</sub>	CEM	9.5	71.8	68	33	21
	HeLa-env <sub>LAI</sub>	HeLa-CD4	11.4	0	0	7.7	0
15	HeLa-env <sub>LAI</sub>	SUP-T1	19.8	2.5	0	18	11
	HeLa-env <sub>LAI</sub>	C8166	15.4	9.7	22	22.3	13

20 Distribution of expression of antigens recognized by mAb PA-3, PA-5, PA-6 and PA-7.

The surface expression of the antigens recognized by the mAb PA-3, PA-5, PA-6 and PA-7 on cell lines known to fuse with HeLa-env<sub>LAI</sub> or HeLa-env<sub>JR-FL</sub> was evaluated by flow cytometry (Figure 1). All of the mAb recognize molecules present on the plasma membrane of PM1 and the related cell line HUT-78. Antigen expression on these two cell lines was equivalent. PA-3 and PA-5 recognize cell surface antigens on both CEM and Sup-T1 cells. Paradoxically, while fusion between HeLa-env<sub>LAI</sub> and CEM cells was inhibited by these mAb, fusion between HeLa-env<sub>LAI</sub> and SUP-T1 was not. Neither PA-6 nor PA-7 recognized CEM or SUP-T1 or significantly inhibited fusion of HeLa-env<sub>LAI</sub> with CEM or Sup-T1. Likewise none

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of the four novel mAb recognized cell surface antigens on HeLa cells or significantly inhibited HeLa-CD4 fusion with HeLa-env<sub>LAI</sub>.

5 A comparison of cell surface staining of two CD4 transfected cell lines and their non-transfected counterparts with mAb PA-3, PA-5, PA-6 and PA-7 (Figure 2) showed that there was no significant increase in expression on the CD4-transfectants. This data is in  
10 complete agreement with ELISA data indicating that none of these four mAb were reactive with human CD4.

**Biochemical analysis of cell surface antigens recognized by mAb PA-3, PA-5, PA-6 and PA-7.**

15 The mAb PA-3, PA-5, PA-6 and PA-7 were used to immunoprecipitate cell surface antigens from PM1 cells by the method described above. This analysis revealed that PA-3 and PA-5 recognized the same antigen (Figure 3). Whether or not both mAb recognized the same epitope  
20 remains to be determined. Two bands of approximate molecular weight 158 Kd and 87 Kd are precipitated with these mAb. When mAb directed against the two chains of LFA-1 (CD11a and CD18) are run along side immunoprecipitations with PA-3, bands of similar size are  
25 precipitated (Figure 4). Thus it is highly likely that PA-3 and PA-5 recognize either CD11a or CD18.

PA-6 and PA-7 both precipitate two proteins of molecular weights approximately 24.8 Kd and 19.1 Kd and thus appear  
30 to recognize the same antigen (Figure 5). However it is unclear whether they recognize the same epitope. When a pan specific HLA class II mAb is used for immunoprecipitation, bands of similar mobilities to those precipitated with PA-6 and PA-7 are resolved (Figure 6).  
35 Thus PA-6 and PA-7 may recognize HLA class II.

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Inhibition of HIV-1<sub>NL4-3</sub> and HIV-1<sub>JR-FL</sub> infection by PA-3, PA-5 and CD4-IgG2.

The assay method was described above. Experiments demonstrated that the antibodies PA-3 and PA-5 inhibit infection by both the laboratory-adapted isolate HIV-1<sub>NL4-3</sub> and the primary macrophage-tropic isolate HIV-1<sub>JR-FL</sub>. Inhibition in these assays was dose-dependent as shown in the following Table 2:

Table 2: Inhibition of HIV-1<sub>NL4-3</sub> and HIV-1<sub>JR-FL</sub> infection by PA-3, PA-5 and CD4-IgG2.

Concentration µg/ml	PA-3		PA-5	
	% Inhibition		% Inhibition	
	of		of	
	HIV-1 <sub>NL4-3</sub>	HIV-1 <sub>JR-FL</sub>	HIV-1 <sub>NL4-3</sub>	HIV-1 <sub>JR-FL</sub>
50.0	90.8	69.5	75.8	57.4
25.0	72.2	49.1	62.0	44.6
12.5	61.8	33.9	58.9	35.4

CD4-IgG2*		
Concentration µg/ml	% Inhibition	
	of	
	HIV-1 <sub>NL4-3</sub>	HIV-1 <sub>JR-FL</sub>
50.0	100.0	95.3
25.0	99.4	75.1
12.5	77.7	62.2

\* Positive control CD4-IgG2 is a fusion protein between CD4 and human IgG2 which neutralizes all strains of HIV-1.

## References

1. Ashorn PA., Berger EA., and Moss B. 1990. Human immunodeficiency virus envelope glycoprotein/CD4-mediated fusion of nonprimate cells with human cells. J. Virol. 64:2149-2156.  
5
2. Broder CC., Dimitrov DS., Blumenthal R., and Berger EA. 1993. The block to HIV-1 envelope glycoprotein-mediated membrane fusion in animal cells expressing human CD4 can be overcome by a human cell component(s). Virology 193:483-491.  
10
3. Clapham PR., Blanc D., and Weiss RA. 1991. Specific cell surface requirements for the infection of CD4-positive cells by human immunodeficiency virus types 1 and 2 and by simian immunodeficiency virus. Virology 181:703-715.  
15
4. Dalglish AG. 1995. HIV and CD26. Nature Medicine 1:881-882.  
20
5. Dragic T., Charneau P., Clavel F., and Alizon M. 1992. Complementation of murine cells for human immunodeficiency virus envelope/CD4-mediated fusion in human/murine heterokaryons. J. Virol. 66:4794-4802.  
25
6. Dragic T., Picard L., and Alizon M. 1995. Proteinase-resistant factors in human erythrocyte membranes mediate CD4-dependent fusion with cells expressing human immunodeficiency virus type 1 envelope glycoproteins. J. Virol. 69:1013-1018.  
30
7. Golding H., Dimitrov DS., and Blumenthal R. 1992.  
35



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LFA-1 adhesion molecules are not involved in the early stages of HIV-1 env-mediated cell membrane fusion. AIDS Res Hum Retroviruses 8:1593-1598.

- 5     8.     Harrington RD. and Geballe AP. 1993. Cofactor requirement for human immunodeficiency virus type 1 entry into a CD4-expressing human cell line. J. Virol. 67:5939-5947.
- 10    9.     Maddon PJ., Dalglish AG., McDougal JS., Clapham PR., Weiss RA., and Axel R. 1986. The T4 gene encodes the AIDS virus receptor and is expressed in the immune system and the brain. Cell 47:333-348.
- 15    10.    Sato AI., Balamuth FB., Ugen KE., Williams WV., and Weiner DB. 1994. Identification of CD7 glycoprotein as an accessory molecule in HIV-1-mediated syncytium formation and cell free infection. J. Immunol. 152:5142-5152.
- 20    11.    Sommerfelt MA. and Asjo B. 1995. Intercellular adhesion molecule 3, a candidate human immunodeficiency virus type 1 co-receptor on lymphoid and monocytoïd cells. J. Gen. Virol. 76:1345-1352.
- 25

**What is claimed is:**

1. An antibody capable of specifically inhibiting the fusion of an HIV-1 envelope glycoprotein\* cell with an appropriate CD4\* cell without cross reacting with the HIV-1 envelope glycoprotein or CD4 and capable of inhibiting infection by one or more strains of HIV-1.
2. A monoclonal antibody of claim 1.
3. A hybridoma cell line producing the monoclonal antibody of claim 2.
4. A chimeric monoclonal antibody of claim 2.
5. A humanized monoclonal antibody of claim 4.
6. A human monoclonal antibody of claim 2.
7. A single chain antibody or an antigen binding antibody fragment of claim 2.
8. A monoclonal antibody capable of competitively inhibiting the binding of the monoclonal antibody of claim 2 to its target molecule.
9. The monoclonal antibody of claim 2, 4, 5, 6, 7, or 8 labelled with a detectable marker.
10. A monoclonal antibody of claim 9 wherein the detectable marker is a radioactive isotope, enzyme, dye or biotin.
11. A pharmaceutical composition comprising the complete or a portion of the monoclonal antibody of claim 2,

4, 5, 6, 7 or 8 and a pharmaceutically acceptable carrier.

- 5 12. A method of inhibiting HIV-1 infection in a subject comprising administering an effective amount of the pharmaceutical composition of claim 11 to the subject.
- 10 13. An isolated nucleic acid molecule encoding the complete or a portion of the light chain protein of the monoclonal antibody of claim 2, 4, 5, 6 or 8.
- 15 14. An isolated nucleic acid molecule encoding the complete of a portion of the heavy chain protein of the monoclonal antibody of claim 2, 4, 5, 6 or 8.
- 20 15. An isolated nucleic acid molecule encoding the single chain antibody of claim 7.
- 25 16. A vector comprising the nucleic acid molecule of claim 13, 14 or 15 operably linked to a promoter of RNA transcription.
- 30 17. A vector comprising the nucleic acid molecules of claims 13 and 14 each operably linked to a promoter of RNA transcription.
- 35 18. A host vector system comprising one or more vectors of claim 16 or 17 in a suitable host cell.
19. A host vector system of claim 18, wherein the suitable host cell is selected from a group consisting of a bacterial cell, an insect cell, a yeast cell or a mammalian cell.

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20. The molecule specifically recognized by the monoclonal antibody of claim 1.

21. A glycolipid molecule of claim 20.

22. A polypeptide molecule of claim 20.

23. An isolated nucleic acid molecule encoding the complete or a portion of the polypeptide of claim 22.

24. A multichain polypeptide molecule comprising the polypeptide of claim 22.

25. A soluble protein comprising a portion of the polypeptide of claim 22 or 24.

26. A pharmaceutical composition comprising an effective amount of the soluble protein of claim 25 to inhibit HIV-1 infection and a pharmaceutically acceptable carrier.

27. A method of inhibiting HIV-1 infection in a subject comprising administering an effective amount of the pharmaceutical composition of claim 26 to the subject.

28. An isolated nucleic acid molecule encoding the complete or a portion of a polypeptide of the multichain polypeptide molecule of claim 24.

29. A vector comprising the nucleic acid molecule of claim 23 or 28 operably linked to a promoter of RNA transcription.

30. A host vector system comprising the vector of claim 29 in a suitable host cell.

5 31. A host vector system of claim 30, wherein the suitable host cell is selected from a group consisting of a bacterial cell, an insect cell, a yeast cell or a mammalian cell.

10 32. A method for identifying inhibitors of HIV-1 infection comprising steps of:

- 15 (a) contacting an effective amount of a compound with a system which contains HIV-1 gp120, HIV-1 gp41 or a fragment thereof with the molecule of claim 20 under conditions permitting formation of a complex between HIV-1 gp120, HIV-1 gp41 or a fragment thereof and the molecule, so as to inhibit such formation; and
- 20 (b) determining the amount of complex formed; and
- (c) comparing the amount determined in step (b) with the control which is without the addition of the compound, a decrease in the complex formation indicating that the compound is capable of inhibiting HIV-1 infection.

25 33. A method of claim 32, wherein the compound is not previously known.

34. The compound identified by claim 33.

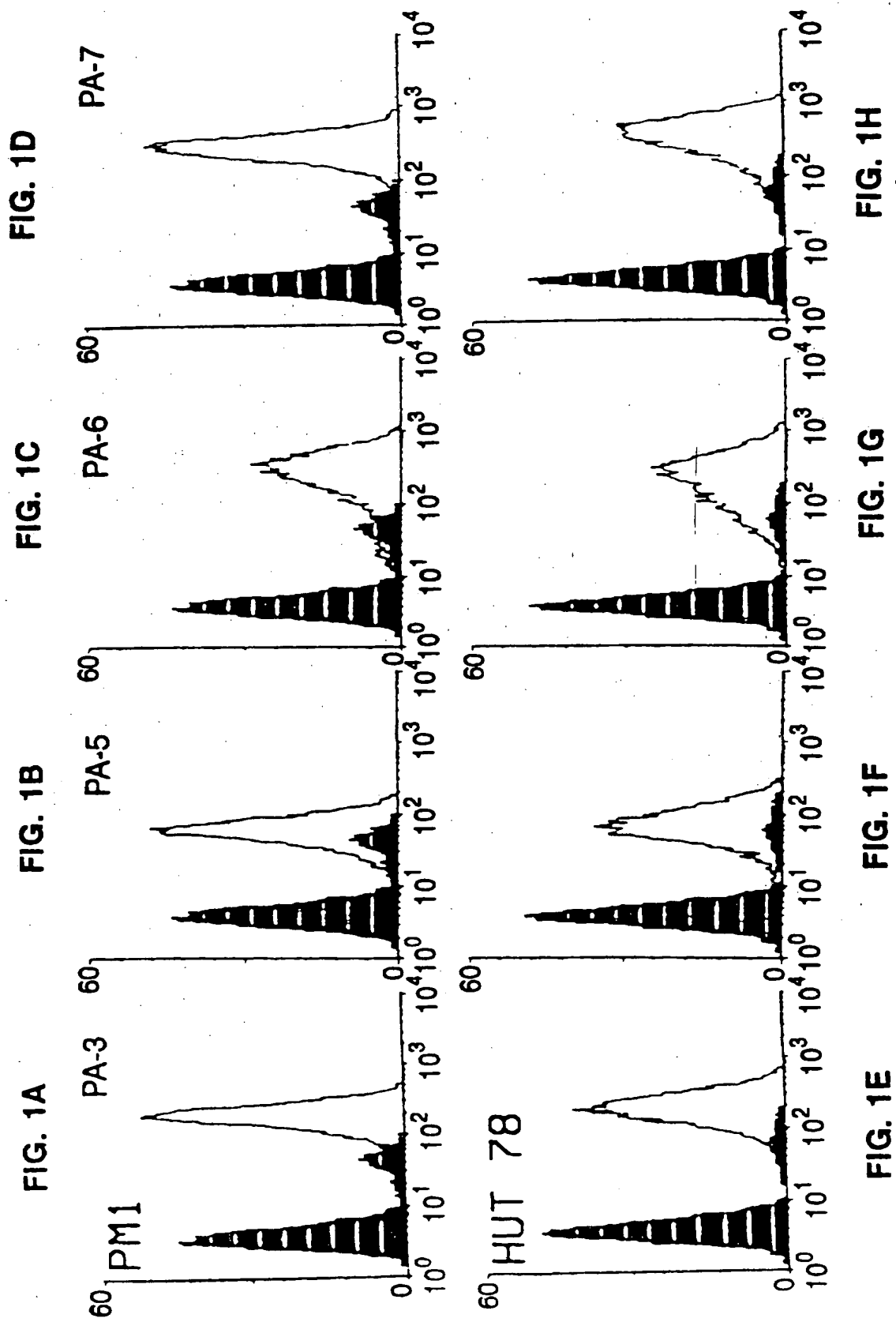
30 35. A pharmaceutical composition comprising the compound identified by the method of claim 32 and a pharmaceutically acceptable carrier.

35 36. A method of inhibiting HIV-1 infection in a subject comprising administering an effective amount of the

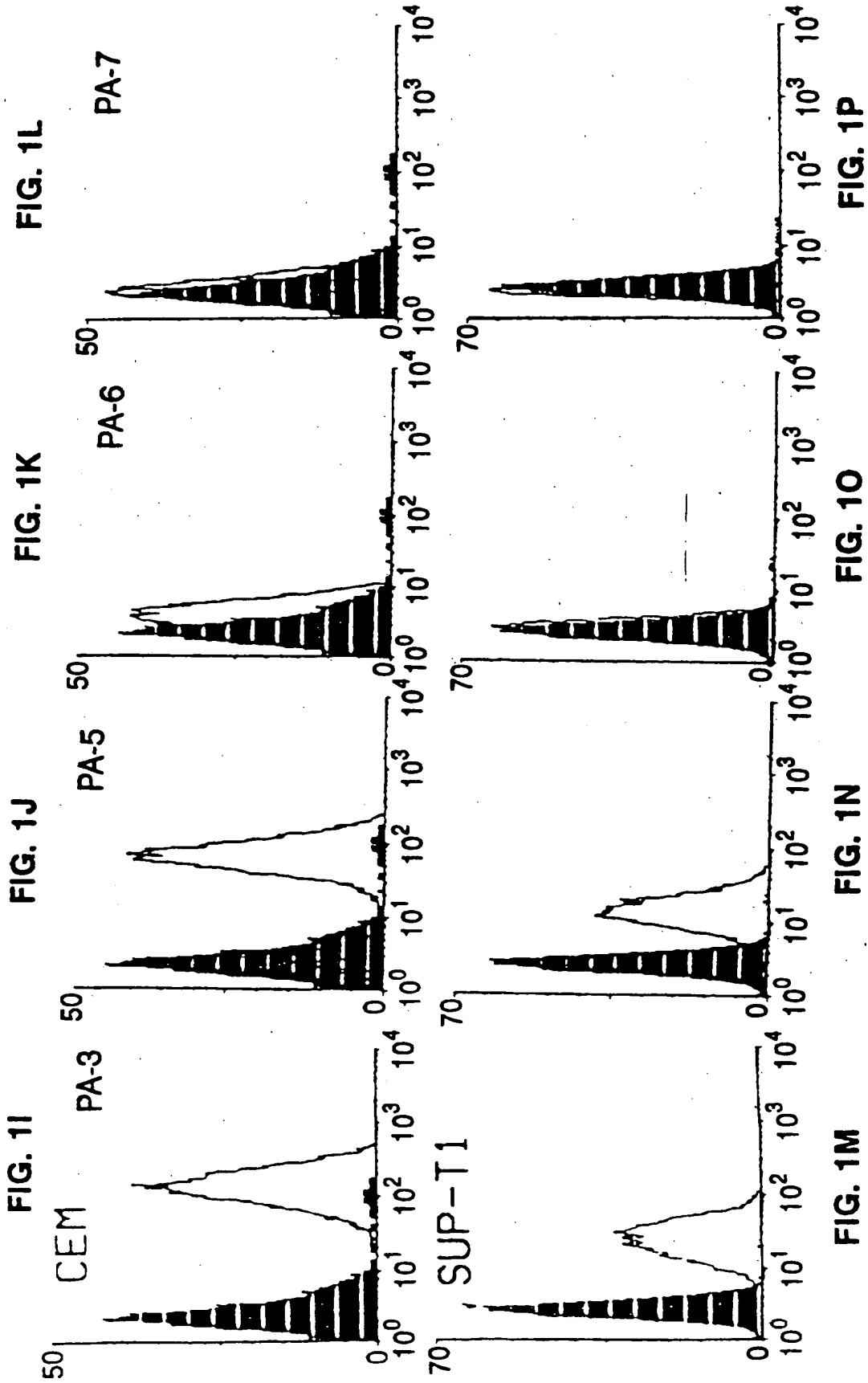
pharmaceutical composition of claim 35 to the subject.

- 5 37. A kit for identifying inhibitors of HIV-1 infection which comprises, in separate compartments:
- (a) purified HIV-1 gp120, HIV-1 gp41 or a fragment thereof; and
  - (b) the molecule of claim 20.
- 10 38. A transgenic nonhuman animal which comprises an isolated DNA molecule encoding the molecule of claim 22 or 24.
- 15 39. The transgenic nonhuman animal of claim 38 further comprising an isolated DNA molecule encoding the full-length or portion of the CD4 molecule sufficient for binding the HIV-1 envelope glycoprotein.

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FIG. 2C

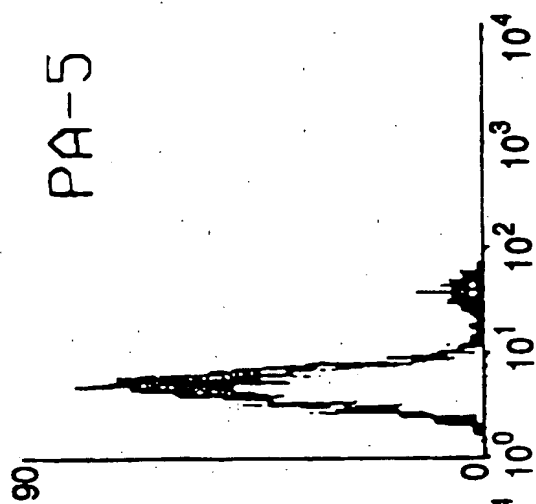


FIG. 2B

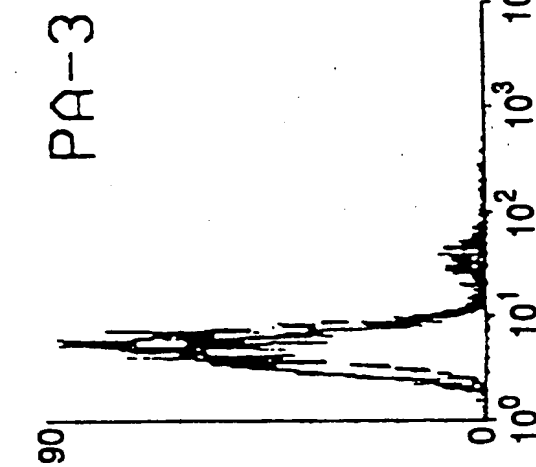


FIG. 2A

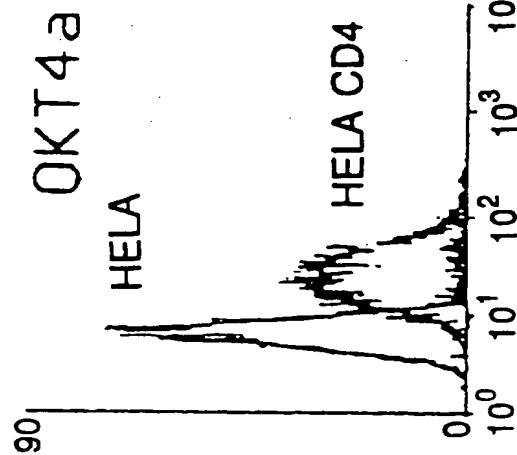


FIG. 2E

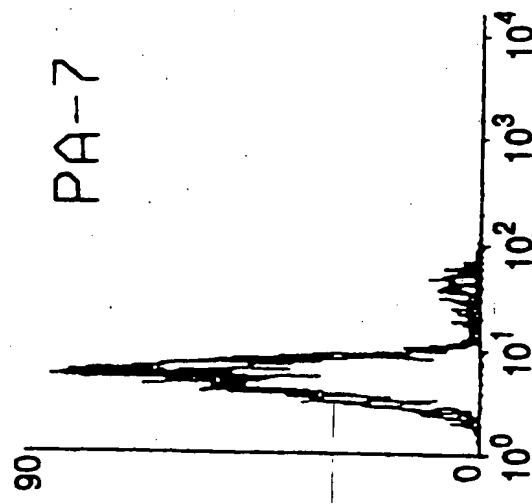
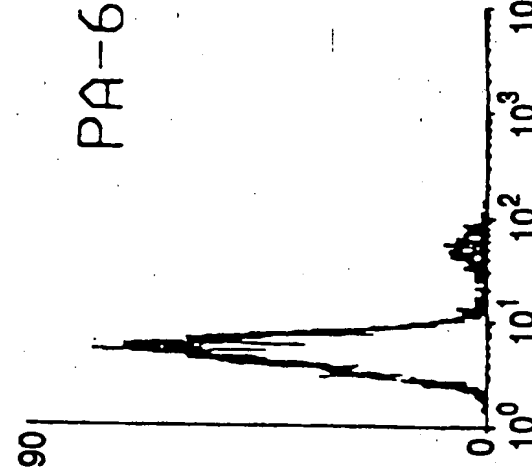


FIG. 2D



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FIG. 2F

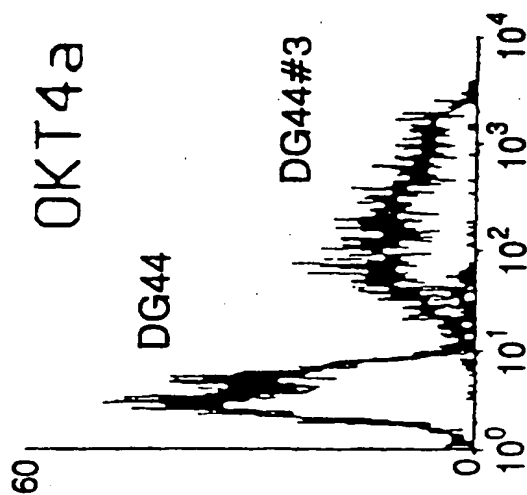


FIG. 2G

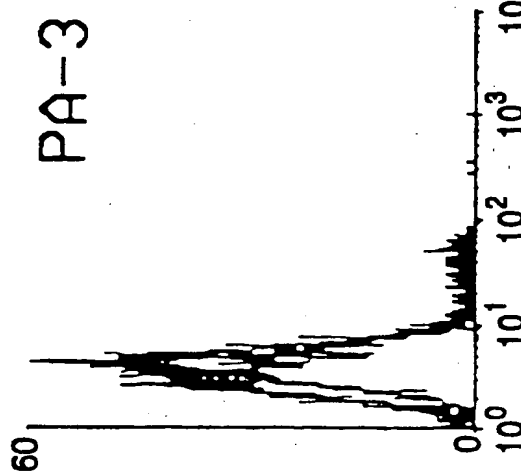


FIG. 2H

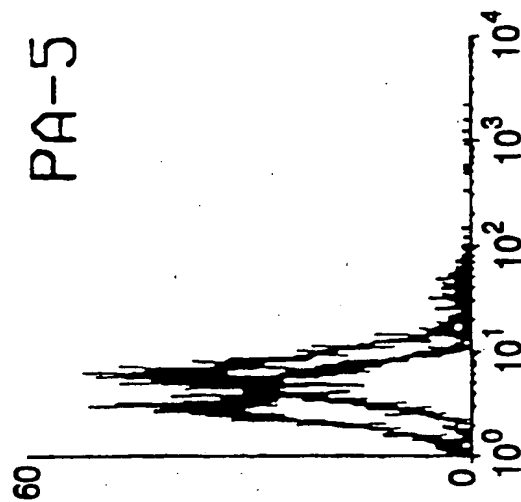


FIG. 2I

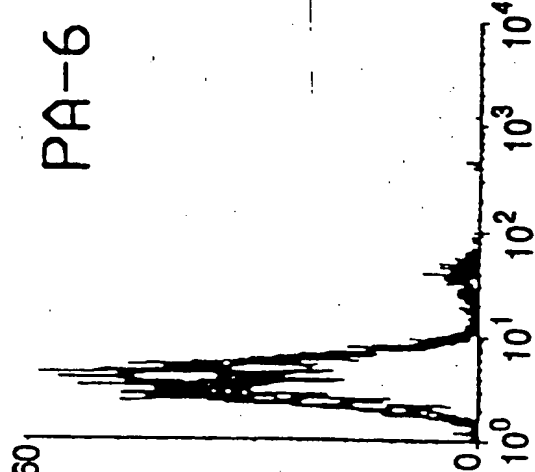
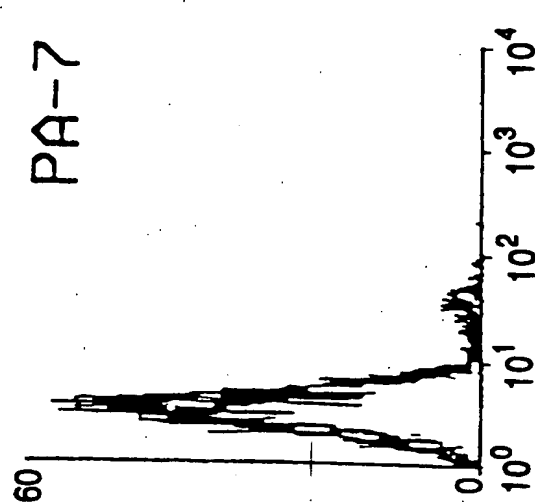
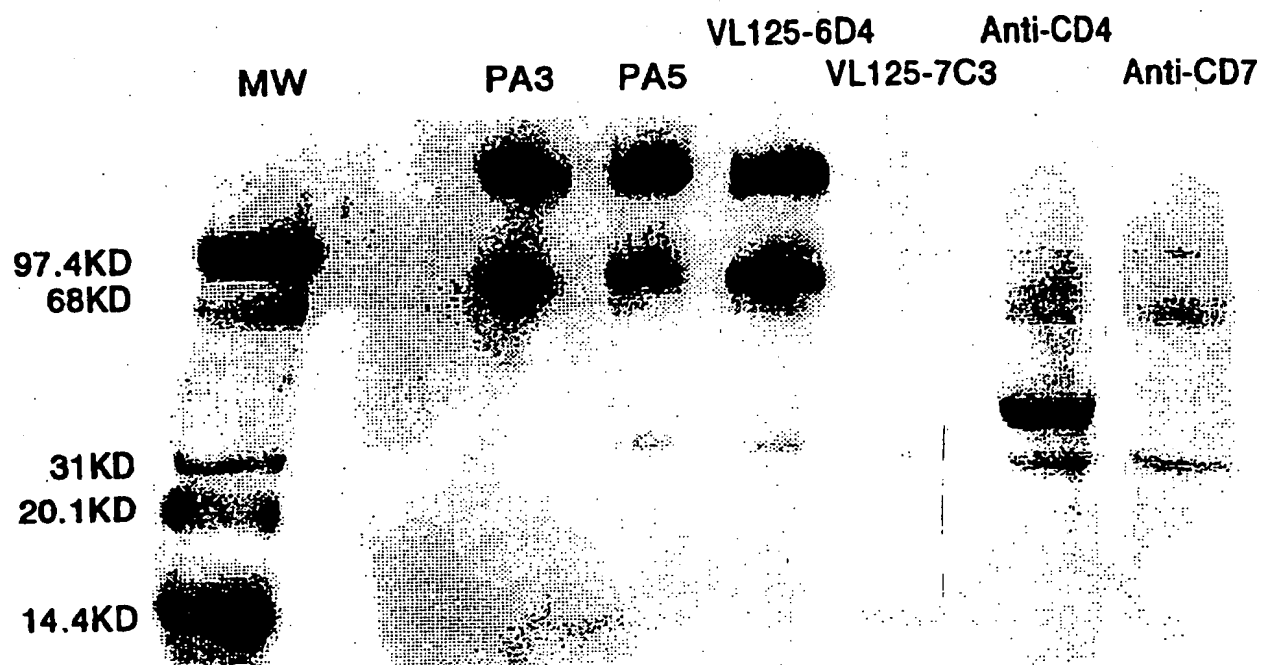


FIG. 2J



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FIG. 3



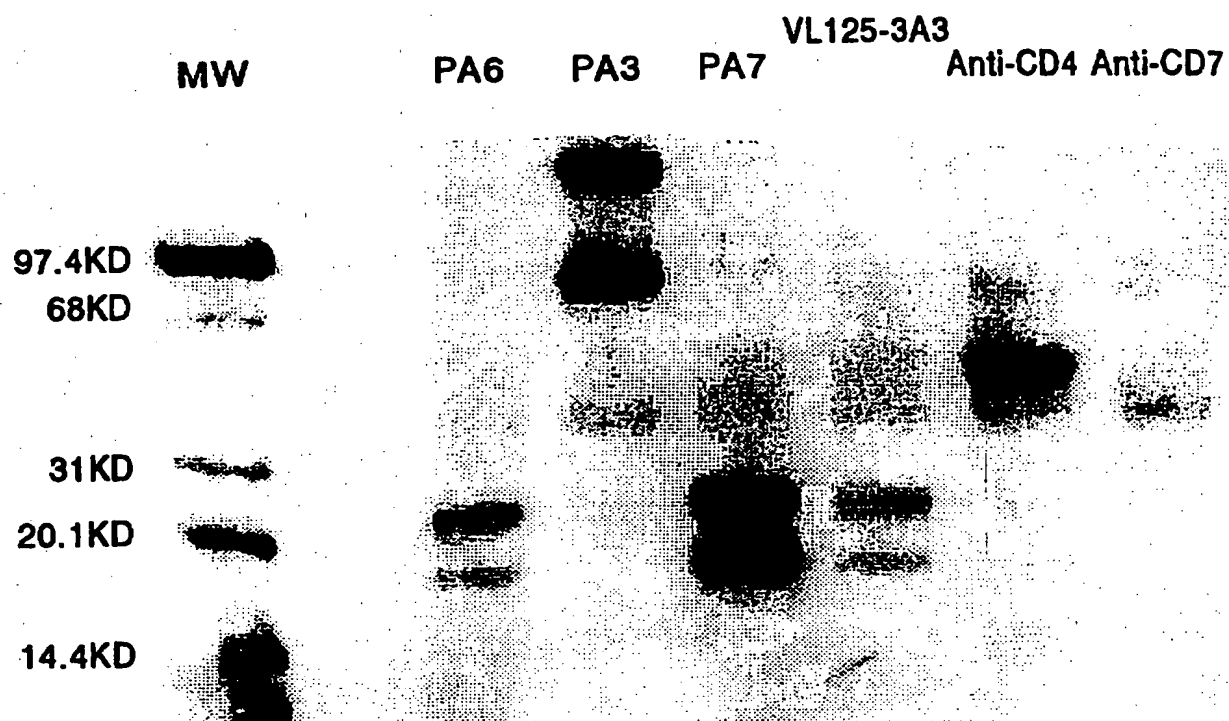
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FIG. 4



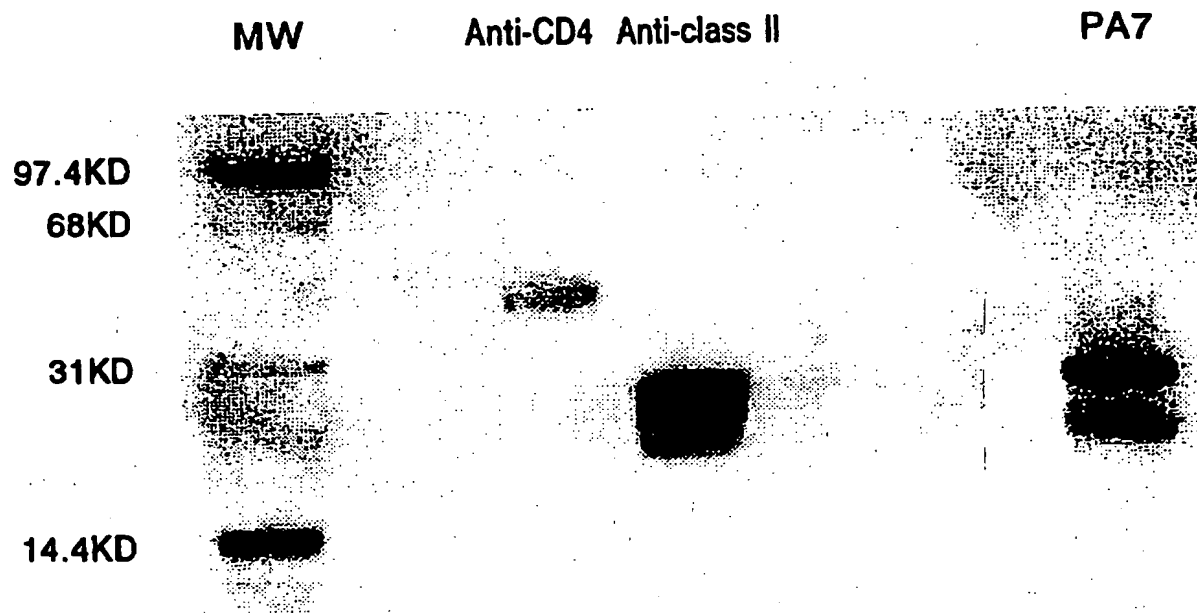
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FIG. 5



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FIG. 6



## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US97/00758

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : A61K 39/395; C12N 5/20, 15/63, 15/12; C07H 21/04

US CL : Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/131.1, 133.3, 135.1, 142.1, 143.1, 192.1 198.1 193.1; 435/7.21, 320, 346; 530/388.7, 388.15, 391.3; 536/23.5; 800/2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS DIALOG, MEDLINE, DERWENT WORLD PATENTS, AIDSLINE, EMBASE HIV antibody, syncytium, inhibition, transgenic

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	VALENTIN et al. The Leukocyte Adhesion Glycoprotein CD18 Participates In HIV induced Syncytia Formation In Monocytoid And T Cells. J. of Immunology. February 1990. Vol 144. pages 934-937, see entire document.	1-3, 11, 32-37 --- 4-39
X ----- Y	BUSSO et al. HIV-Induced Syncytium Formation Requires The Formation Of Conjugates Between Virus-Infected And Uninfected T-Cells In Vitro. AIDS. December 1991. Vol. 5. pages 1425-1432, see entire document.	1-3, 11, 32-37 ----- 4-12
X --- Y	SATO et al. Anti-CD7 Reagents Inhibit HIV-1 Induced Syncytium Formation. International Conference on AIDS. 1992. Vol. 8I. PA5 PoA 2017, see entire article.	1-3, 11, 32-37 --- 4-39

☒ Further documents are listed in the continuation of Box C.
 ☐ See patent family annex.

* Special categories of cited documents:	*T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be of particular relevance	*X*	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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*O* document referring to an oral disclosure, use, exhibition or other means		
*P* document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

20 MARCH 1997

Date of mailing of the international search report

09 JUN 1997

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## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ----- Y	HATTORI et al. Involvement of Tryptase-Related Cellular Protease(s) in Human Immunodeficiency Virus Type 1 Infection. FEBS Letters. May 1989. Vol. 248. pages 48-52, see entire document.	1-3, 11, 32-37, 20, 22, 26, 32-37 --- 4-39
X ----- Y	HILDRETH et al. Involvement Of A Leukocyte Adhesion Receptor (LFA-1) In HIV-Induced Syncytium Formation. Science. 02 June 1989. Vol 244. pages 1075-1078, see entire document.	1-3, 11, 32-35 --- 4-39
Y	ATTANASIO et al. Anti-Idiotypic Antibody Response To Monoclonal Anti-CD4 Preparations In Nonhuman Primate Species. J. of Immunology 15 January 1991. Vol 146. pages 507-514, see entire document.	8, 20-39
Y	SCHANBERG et al. Characterization Of Human CD7 Transgenic Mice. J. of Immunology. 01 September 1995. Vol. 155. pages 2407-2418, see entire document.	20-39
Y	QUEEN et al. A Humanized Antibody That Binds To The Interleukin 2 Receptor. Proceedings of the National Academy of Science USA. December 1989. Vol. 86. pages 10029-10033, see entire document.	5-39
Y	US 5,071,964 (DUSTIN et al) 10 December 1991. see entire document.	21-39
Y	US 5,091,513 (HUSTON et al.) 25 February 1992. see entire document.	7
Y	WANG et al. Deletion Of T lymphocytes In Human CD4 Transgenic Mice Induced by HIV-gp120 and gp120-Specific Antibodies From AIDS Patients. Eur. J. Immunol. 1994. Vol. 24. pages 1553-1557, see entire document.	39



A. CLASSIFICATION OF SUBJECT MATTER:

US CL :

424/131.1, 133.3, 135.1, 142.1, 143.1, 192.1 198.1 193.1; 435/7.21, 320, 346; 530/388.7, 388.15, 391.3; 536/23.5;  
800/2